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Utilization of the indirect lysosome targeting pathway by lysosome-associated membrane proteins (LAMPs) is influenced largely by the C-terminal residue of their GYXXΦ targeting signals

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SUMMARY

A systematic study was conducted on the requirements at the C-terminal position for the targeting of LAMPs to lysosomes, examining the hypothesis that a bulky hydrophobic residue is required. Mutations deleting or replacing the C-terminal valine with G, A, C, L, I, M, K, F, Y, or W were constructed in a reporter protein consisting of the lumenal/extracellular domain of avian LAMP-1 fused to the transmembrane and cytoplasmic domains of LAMP-2b. The steady-state distribution of each mutant form in mouse L-cells was assessed by quantitative antibody binding assays and immunofluorescence microscopy; efficiency of internalization from the plasma membrane and delivery to the lysosome were also estimated. It is found that (a) only C-terminal V, L, I, M, and F mediated efficient targeting to lysosomes, demonstrating the importance hydrophobicity and an optimal size of the C-terminal residue in targeting; (b) efficiency of lysosomal targeting generally correlated with efficiency of internalization; and (c) mutant forms that did not target well to lysosomes showed unique distributions in cells rather than simply default accumulation in the plasma membrane. Interactions of the targeting signals with adaptor subunits were measured using a yeast two-hybrid assay. The results are consistent with the hypothesis that trafficking of LAMP forms in cells through the indirect pathway is determined by the affinities of their targeting signals, predominantly for the μ2 and μ3 adaptors involved at plasma membrane and endosomal cellular sorting sites, respectively.

Key words: LAMP, Lysosome associated membrane protein, Lysosome, Protein targeting, Endocytosis, Adaptor

INTRODUCTION

LAMP-1 and LAMP-2, lysosome-associated membrane proteins, are structurally similar proteins derived from separate but evolutionarily related genes. The LAMP-1 gene encodes a single transcript (Zot and Fambrough, 1990), while the LAMP-2 gene encodes several transcripts (for LAMP-2a, LAMP-2b, and LAMP-2c) (Gough et al., 1995; Hatem et al., 1995; Konecki et al., 1995) with alternatively spliced 3′ exons. LAMP-1 and LAMP-2a, -2b, and -2c have large, extensively glycosylated lumenal domains with four disulfide bridges, a single transmembrane domain, and a short cytosolic tail domain (reviewed by Fukuda, 1991). The LAMP-1 cytosolic domain is invariant from chickens to mammals, but due to alternative splicing, the LAMP-2 family members have different cytosolic domains as well as different transmembrane domains.

Newly synthesized LAMPs utilize two cellular pathways to reach the lysosome: a direct pathway in which the LAMPs are targeted directly to a late-endosome/lysosome compartment from the trans-Golgi network and an indirect pathway in which the LAMPs move from the trans-Golgi network to an early endosome/plasma membrane compartment first and then are internalized and delivered to lysosomes (reviewed by Peters and von Figura, 1994; Sandoval and Bakke, 1994; Hunziker and Geuze, 1996). Targeting of LAMPs to lysosomes is dependent upon the C-terminal five residues of the cytosolic tail, which conform to the sequence -G-Y-X-X-Φ, where Φ is a hydrophobic residue (Guarnieri et al., 1993; Höning and Hunziker, 1995). The LAMP-1 cytosolic tail has been explored to a limited extent by site-directed mutagenesis and its targeting role has been shown to depend on the presence and positions of the glycyl and tyrosyl residues (Williams and Fukuda, 1990; Hunziker et al., 1991; Harter and Mellman, 1992; Guarnieri et al., 1993; Höning and Hunziker, 1995), the distance of the signal from the membrane (Rohrer et al., 1996), and the identity of the C-terminal residue (Williams and Fukuda, 1990; Guarnieri et al., 1993; Gough and Fambrough, 1997). Mutations within the cytosolic tail may either disrupt lysosomal targeting altogether, resulting in accumulation at the

cell surface, or alter the relative use of the direct versus indirect pathway of lysosomal targeting (Williams and Fukuda, 1990; Harter and Mellman, 1992; Guarnieri et al., 1993; Akasaki et al., 1995; Höning and Hunziker, 1995).

While LAMPs are predominantly lysosomal, they can also be found cycling between the endosomal compartments and the plasma membrane (Lippincott-Schwartz and Fambrough, 1986, 1987; Furuno et al., 1989a,b; Akasaki et al., 1993). The steady-state distribution of LAMPs among cellular membranes is dependent largely upon the C-terminal cytosolic domain. Previously, we demonstrated that the three different LAMP-2 cytosolic tails result in different steady-state distributions of LAMP-1/LAMP-2 chimeric proteins, most notably different levels of expression at the cell surface (Gough and Fambrough, 1997). The LAMP-2c tail resulted in the lowest level of cell surface expression (6%), while the LAMP-2b tail resulted in the highest level (25%). These differences in cell surface expression depended primarily upon the identity of the hydrophobic residue (V, L or F) at the C terminus (Gough and Fambrough, 1997). It follows that the cellular trafficking machinery can distinguish among these C-terminal hydrophobic residues of LAMP proteins. In the present study, we extended these observations by systematically replacing the C-terminal residue of LAMP-2b with aminoacyl residues of varying hydrophobicity and side chain size and assessing targeting to lysosomes, cell surface expression, and internalization and delivery to lysosomes of surface-expressed LAMP molecules. Additionally, we tested the various C-terminal residues in the LAMP targeting signals for interaction with the medium (μ) subunit of adaptor complexes known to mediate LAMP trafficking.

MATERIALS AND METHODS

Construction of chimeras

The coding sequences of avian LAMP-1 (formerly called LEP100; Lippincott-Schwartz and Fambrough, 1987; Fambrough et al., 1988) and the LAMP chimeras and point mutants were cloned into the vector pCB6, which contains the human cytomegalovirus promoter for expression in mammalian cells and the neomycin resistance gene for selection in G418 containing medium. The chimeras and point mutants were comprised of the lumenal domain of avian LAMP-1 and the transmembrane and cytosolic tails of the avian LAMP-2 isoforms. The LAMP-1/LAMP-2b chimera as well as the LAMP-1/LAMP-2a and LAMP-1/LAMP-2c chimeras were described previously (Gough and Fambrough, 1997). The point mutants in the C-terminal position were all made in the context of the LAMP-1/LAMP-2b chimera, using the Quick Change kit (Stratagene, La Jolla, CA). The nucleotide sequences encoding the complete transmembrane and cytosolic tail of each chimera were confirmed by dye terminator automated sequencing with the ABI Prism 310 Genetic Analyzer. The Quick Change primers in the trityl-on form were purchased from GeneMed Synthesis Inc. (San Francisco, CA) and purified through oligonucleotide purification cartridges.

Cell lines and culture

Plasmids (5 μg DNA/60 mm dish of 60% confluent cells) were introduced into mouse L cells with LIPOFECTIN (Life Technologies, Gaithersburg, MD) and OPTIMEM medium. Stable cell lines were selected in Dulbecco's minimal essential medium (DMEM) with 10% fetal calf serum and 1% gentamycin, supplemented with 400 μg/ml G418. Each cell line consisted of a set of pooled clones. Expression of chimeric LAMPs was induced by treatment of the transfected cells with 10 mM butyrate for 48±2 hours. Cells treated with cycloheximide were induced with 10 mM butyrate for 44 hours, then 75 μg/ml cycloheximide was added for the remaining 4 hours of induction time.

Chimera detection and quantification

The LAMP chimeras expressed in mouse L cells were detected with a monoclonal antibody (Mab-CV24) against avian LAMP-1 lumenal/extracellular domain (Lippincott-Schwartz and Fambrough, 1986), which does not cross-react with mouse LAMPs. Mab-CV24 can be radiolabelled with ^{125}I without loss of activity, permitting direct detection and quantification of chimeras expressed by the transfected cells.

Immunofluorescent labeling of fixed, permeabilized cells was performed as described by Gough and Fambrough (1997) with 2 μg/ml Mab-CV24 in Hanks' balanced salt solution, 20 mM Tris-Cl, pH 7.5 and 2% horse serum (H/T/HS) supplemented with 0.1% saponin. For double labeling experiments to test for lysosomal targeting, fluorescein isothiocyanate (FITC)-conjugated Mab-CV24 was used to detect the LAMP chimeras and Cy3-conjugated Mab-1D4B (Chen et al., 1985) was used to detect endogenous mouse LAMP-1. For maximal labeling of the transfected cells with Mab-1D4B antibody in double-labeling experiments, Cy3-Mab-1D4B was applied first for 2 hours, then after rinsing the cells, FITC-Mab-CV24 was applied for 1 hour. To test for accumulation in endosomes, cells were double-labeled with Cy3-Mab-CV24 and anti-mouse transferrin receptor antibody R17 217 (Lesley et al., 1984), which was visualized with FITC-labeled goat anti-rat secondary antibody. Where indicated, the cells were preincubated in 5 μg/ml unlabeled Mab-CV24 prior to permeabilization; this pretreatment blocked surface LAMP sites and allowed subsequent selective labeling of intracellular sites with directly-conjugated FITC-Mab-CV24 or Cy3-Mab-CV24. Confocal images were collected on a Zeiss LSM confocal microscope and processed with Adobe Photoshop.

Antibody binding assays

The transfected protein expressed at the cell surface was quantified by iodinated antibody binding to permeabilized and intact (non-permeabilized) fixed cells as described by Gough and Fambrough (1997). Briefly, Mab-CV24 was iodinated by the Iodogen method (Salicinski et al., 1981). Cells were plated in duplicate or triplicate in 24-well dishes at 1×10^5 cells/well, induced the following day with 10 mM butyrate and used for binding experiments after 48 hours. Cells were fixed in buffered 1% formaldehyde, rinsed, then incubated with 2 μg/ml ^{125}I-Mab-CV24 in H/T/HS with or without 0.1% saponin. Specific binding was calculated by subtracting the non-specific cpm from total cpm for both permeabilized and intact conditions. Non-specific binding was determined by (a) measuring the amount of ^{125}I-Mab-CV24 bound in the presence of 25- to 50-fold excess unlabeled Mab-CV24 with or without 0.1% saponin to permeabilize the cells or (b) determining the amount of ^{125}I-Mab-CV24 bound to butyrate-treated, non-transfected L cells with or without 0.1% saponin. Each experimental condition was assayed in duplicate or triplicate. The expression level for each pool of stably transfected cells was estimated using the specific cpm ^{125}I-Mab-CV24 bound in the presence of saponin. The fraction of LAMP molecules present at the cell surface was calculated by dividing the specific cpm ^{125}I-Mab-CV24 bound in the absence of saponin by the specific cpm bound in the presence of saponin.

Antibody internalization assays

The internalization of LAMP molecules was detected using two methods: (1) uptake of Mab-CV24 bound to the surface LAMP molecules with subsequent visualization by indirect immunofluorescence microscopy and (2) measurement of the amount of ^{125}I-Mab-CV24 bound to LAMP molecules at the cell surface that became resistant to removal from the surface by acid stripping after 30 minutes at 37°C.

For the first method, stable cell lines were plated onto coverslips and induced with butyrate for 44 hours. The medium was replaced with Hepes-buffered DMEM, pH 7.2, containing 10-20 μg Mab-CV24 and cells were placed in an incubator at 37°C, 5% CO_2 for 30 minutes to 3 hours. After the incubation period, the cells were washed in H/T/HS and fixed in 1% formaldehyde and permeabilized in 0.1% saponin. Mab-CV24 was detected with FITC-anti-mouse secondary antibody. Lysosomes were identified by colabeling the cells with Cy3-Mab-1D4B in the presence of an excess of an irrelevant monoclonal antibody to block unbound sites on the anti-mouse secondary.

For the quantitative internalization assay performed with ^{125}I-Mab-CV24, stable cell lines were plated in duplicate in 6-well plates at 5×10^5 cells/well and induced with butyrate for 48 hours. Cells were incubated for 20 minutes at room temperature in H/T/HS to decrease non-specific binding. Cells were then cooled on wet ice, washed once with cold H/T/HS, and incubated for 30 minutes with 5 μg/ml ^{125}I-Mab-CV24 in H/T/HS on ice. The cells were washed in 2 baths of ice-cold Hanks' balanced salt solution buffered with 20 mM Tris for 5 minutes each to remove unbound antibody. Cells were either treated with ice-cold Hepes-buffered DMEM or incubated at 37°C in prewarmed Hepes-buffered DMEM for 30 minutes. The medium was collected. The surface antibody was stripped with ice-cold 0.1 M acetic acid/1.5 M NaCl twice for 15 minutes each and collected. Finally, the cells were extracted in 1 N NaOH at room temperature. The media, stripping solutions, and cell extracts were counted in a gamma counter. Non-specific binding was determined from butyrate-treated non-transfected L cells handled identically. After corrections for non-specific binding, the percent of cell-associated antibody that was resistant to low pH stripping was calculated by dividing cpm in the cell extracts by total cpm present in the media, stripping solutions, and cell extracts. Cells kept on ice for the entire experiment were considered the zero time point, cells warmed to 37°C were considered the 30 minute time point. Internalized antibody was calculated by subtracting the time zero cell-associated cpm resistant to removal by low pH from the 30 minute cell-associated cpm.

Yeast two-hybrid assays

Overlapping oligonucleotides encoding the TGN38 cytosolic linker/LAMP-2b tail sequences were ligated using *Pst*I and *Eco*RI sites into the pGBT9 vector. The fidelity of the inserted sequences were confirmed by dye terminator automated sequencing with the ABI Prism 310 Genetic Analyzer. The *Saccharomyces cerevisiae* strain HF7c (MAT**a**, ura3-52, *HIS3*-200, lys 2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS2::GAL1-*HIS3*, URA3::(GAL4 17-mers)$_3$-CYC1-lacZ) (Clontech) was maintained on YPD agar plates. Transformation was done by the lithium acetate procedure as described in the instructions for the MATCHMAKER two-hybrid kit (Clontech). For colony growth assays, HF7c transformants were streaked on plates lacking leucine, tryptophan and histidine and allowed to grow at 30°C, usually for 3-4 days, until colonies were large enough for further assays. Quantitative assays for growth in the presence of varying concentrations of 3AT (3-amino-1,2,4-triazole, Fluka Chemie AG, Buchs, Switzerland) were performed as described (Aguilar et al., 1997).

RESULTS

Chimeras in which the lumenal domain of avian LAMP-1 replaced the lumenal domain of avian LAMP-2b were constructed to allow the proteins to be recognized by the monoclonal antibody Mab-CV24 against the lumenal domain of avian LAMP-1. Ten mutations of the C-terminal aminoacyl residue were made as well as a construct in which the C-terminal residue was deleted by insertion of a stop codon (Table 1). The aminoacyl residues that were placed at the C-terminal position in the LAMP chimeras represented a range of hydrophobicities as well as accessible surface areas (Table 1). Each of the constructs was transfected into mouse L cells, stably transfected cells were pooled, and expression was induced with butyrate.

Localization of chimeric LAMPs at the cell surface is strongly influenced by the C-terminal residue

The LAMP chimeras with different C-terminal residues accumulated in the plasma membrane at vastly different levels. Levels of cell surface expression were quantified by binding of ^{125}I-Mab-CV24 to intact and permeabilized cells (Fig. 1). Based on this criterion, the chimeras can be grouped into two categories: (1) those chimeras with cell surface expression equal to or less than the chimera that contains the LAMP-2b cytosolic tail (C-terminal valine), and (2) those chimeras with higher levels of cell surface expression. Each of the category 1 chimeras has a C-terminal residue that is also found at the C terminus of one or another naturally occurring membrane

Table 1. Properties of the C-terminal residue of the LAMP-1/LAMP-2b chimeras

C-terminal residue	Accessible surface area (Å^2)	Hydrophobicity based on OMH scale‡	Hydrophobicity based on Kyte-Doolittle§	Lysosomal targeting sequence of lysosomal membrane proteins¶
Glycine	75	–0.67	–0.4	-
Alanine	115	–0.40	1.8	-
Δ Serine*	115	–0.55	–0.8	-
Cysteine	135	0.17	2.5	-
Valine	155	0.91	–1.3	LAMP-2b (GYQSV)
Leucine	170	1.22	3.8	LAMP-2c (GYQTL)
Isoleucine	175	1.25	4.5	LAMP-1 (GYQTI)
Methionine	185	1.02	1.9	LIMP-I (GYEVM)
Lysine	200	–0.67	–3.9	-
Phenylalanine	210	1.92	2.8	LAMP-2a (GYEQF)
Tyrosine	230	1.67	–1.3	-
Tryptophan	255	0.50	–0.9	-

*Δ Serine is a deletion of the C-terminal residue, resulting in a serine at the C terminus.
‡Sweet and Eisenberg, 1983.
§Kyte and Doolittle, 1982.
¶Righthand column lists a naturally occurring lysosomal membrane protein that carries the specified C-terminal residue, followed in parentheses by the sequence of its -G-Y-X-X-Φ targeting signal.

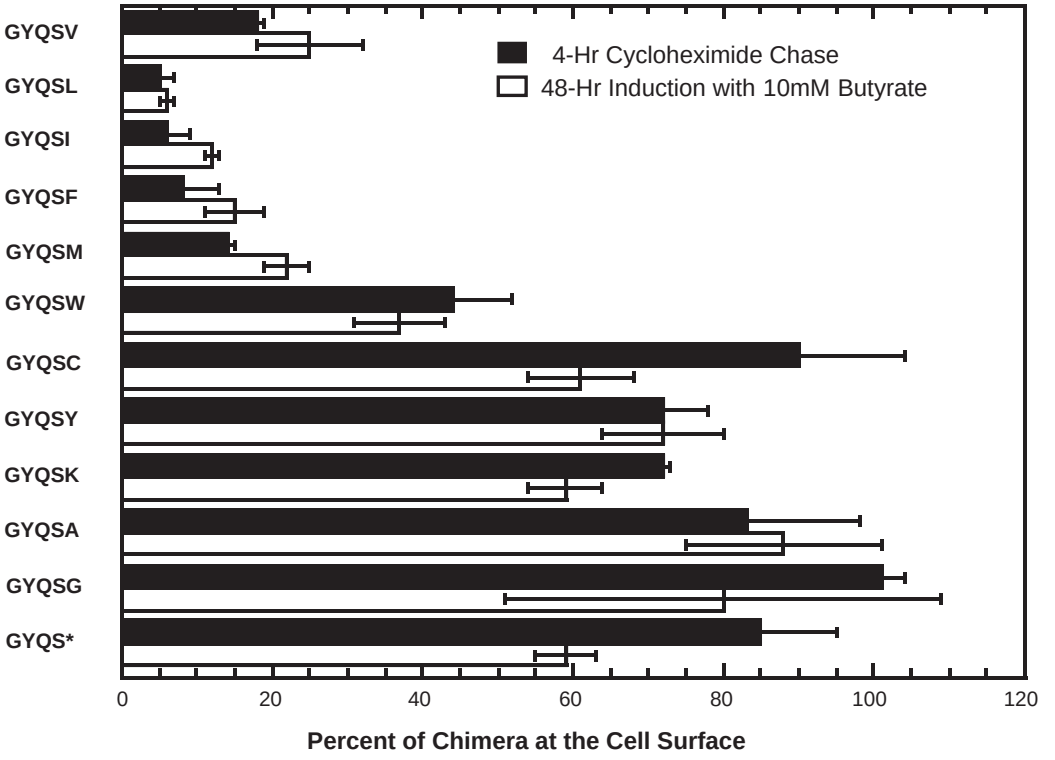

Fig. 1. Quantification of LAMP chimeras at the cell surface. Surface and total sites were measured by ^{125}I-Mab-CV24 binding to induced cells in the absence or presence of saponin to permeabilize the cells. Non-specific binding was determined by binding in the presence of 50-fold excess of unlabeled Mab-CV24. Cells were induced for either 48 hours or 44 hours followed by 4 hours of cycloheximide treatment. Each chimera was assayed in triplicate in at least two independent experiments. The average and standard deviation are shown.

protein targeted to lysosomes via a -G-Y-X-X-Φ C-terminal signal (Table 1).

Several lines of evidence indicate that the differences in cell surface expression levels are not due to saturation of the intracellular targeting machinery. Uthayakumar and Granger (1995) showed that very high overexpression altered the targeting of LAMPs in only one of three cell lines they studied. Similarly, we found that the subcellular distribution of endogenous LAMPs in mouse L cells was unaffected even when the cells expressed 1×10^6 molecules of exogenous LAMP-2 (Gough and Fambrough, 1997), using expression conditions identical to those employed in the present study. Furthermore, in the present study each cell line was a pooled population of transfected cells, so the data are averaged over a range of expression levels, few if any cells expressing as much as 1×10^6 molecules of exogenous LAMP.

Quantification of cell surface expression of the chimeric LAMPs was carried out with or without pretreatment of cells with cycloheximide for 4 hours to allow LAMP proteins in the biosynthetic pathway to reach their terminal destinations (Fig. 1). Changes in surface expression in the presence of cycloheximide were judged significant only if standard deviations were non-overlapping. For those chimeras with a C-terminal residue found at the C terminus of naturally occurring lysosomal membrane proteins, the level of cell surface expression was either decreased (C-terminal M and I) or statistically unchanged (C-terminal L, F, and V) by the cycloheximide pretreatment. The decrease in surface levels may be due in part to internalization of proteins utilizing the indirect path via the plasma membrane to lysosomes. The chimeras with C-terminal cysteine, glycine and stop showed increased cell surface accumulation to greater than 80% of total molecules after cycloheximide treatment, suggesting that there was a significant fraction of chimera molecules in transit through the biosynthetic pathway to plasma membrane. To explore this matter further, cells expressing the C-terminal cysteine chimera were analyzed by immunofluorescence microscopy with and without cycloheximide pre-treatment (Fig. 2). Without cycloheximide, cells showed a nuclear ring of immunofluorescence, consistent with the chimera being in the biosynthetic pathway; this labeling pattern was not seen in cells pre-treated with cycloheximide. The levels of cell surface expression of chimeras having C-terminal alanine, tryptophan, tyrosine and lysine were little changed by cycloheximide treatment.

The C-terminal residue controls the accumulation of the LAMP chimeras in the lysosome

To determine the ability of LAMP chimeras with different C-terminal residues to accumulate in lysosomal membranes, the transfected cells were analyzed by confocal immunofluorescence microscopy. Some cells of each line were treated with cycloheximide for 4 hours in order to deplete them of newly synthesized LAMP molecules that might be in the biosynthetic pathway. LAMP chimeras with C-terminal residues found on naturally occurring lysosomal membrane proteins, i.e. valine, phenylalanine, isoleucine, leucine and methionine, all colocalized with the endogenous LAMP-1 in lysosomes (Figs 3 and 4). While the intensities of the immunofluorescence labeling of individual lysosomes were sometimes different, the overall patterns of immunolabeling were identical for these chimeras and the endogenous LAMP-1, indicating that the intracellular location of each of these five chimeras was predominantly lysosomal.

To analyze the intracellular locations of the C-terminal cysteine, tyrosine and tryptophan mutants, the cell surface sites for Mab-CV24 were blocked with unlabeled antibody prior to

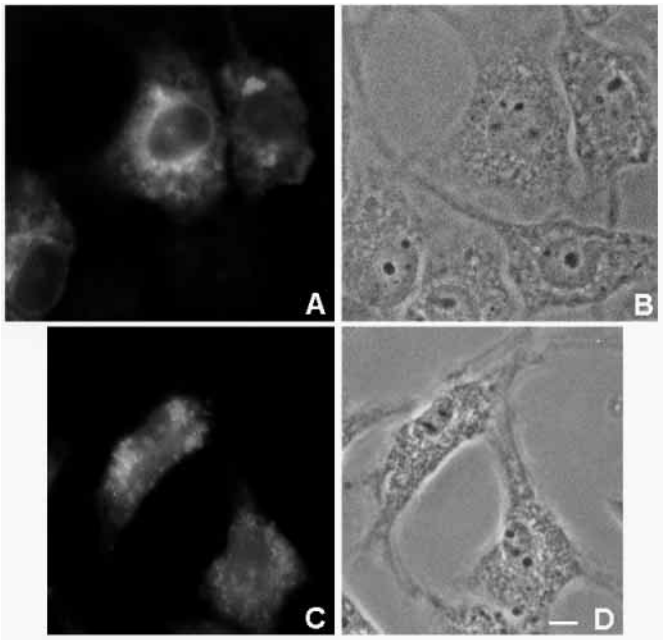

Fig. 2. Immunofluorescence of cells expressing the chimera with C-terminal cysteine with and without cycloheximide treatment. (A and B) Cells were induced with butyrate, fixed, permeabilized and labeled with Cy3-Mab-CV24. (C and D) Cells were induced with butyrate, then treated with cycloheximide for 4 hours. After fixation, the cells in C and D were incubated with Mab-CV24 to block surface sites of the chimera, then were permeabilized and labeled with Cy3-Mab-CV24. (C and D) Phase contrast images corresponding to the cells in A and B, respectively. Bar, 10 μm.

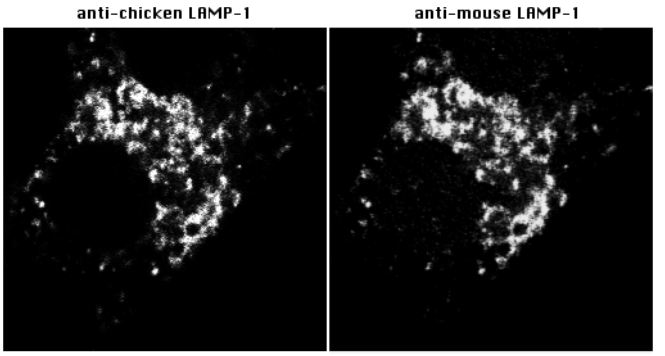

Fig. 3. Colocalization of intracellular LAMP-1/LAMP-2b chimera with mouse LAMP-1. Cells were induced with butyrate, then treated with cycloheximide for 4 hours. After fixation and permeabilization, the cells were labeled with FITC-Mab-CV24 and Cy3-Mab-1D4B, antibodies to the LAMP chimera and endogenous LAMP-1, respectively. Confocal images were collected at the level of the nucleus.

permeabilization so that surface labeling did not mask the distribution of internal sites. For chimeric LAMP with tyrosine at the C terminus, the intracellular labeling pattern was coincident with the endogenous LAMP-1 (Fig. 5), suggesting that the targeting signal ending in tyrosine was capable of directing some of the LAMP chimera to the lysosome, albeit inefficiently since less than 30% of the molecules were intracellular (Fig. 1). Cells expressing the chimeric LAMP with cysteine at the C terminus only showed partial overlap between intracellular chimera and endogenous LAMP-1 (Fig. 5). In these cells there was also a population of vesicles that did not contain LAMP-1 but did contain the chimera, suggesting that the chimera with C-terminal cysteine was not recognized well as a lysosomal targeting signal. One population of intracellular vesicles in which the C-terminal cysteine chimera might reside is early endosomes. To explore this possibility further, cells were double-labeled with an antibody against mouse transferrin receptors (Lesley et al., 1984) and Mab-CV24, and some colocalization of the antibodies was seen (data not shown). Finally, the chimera with tryptophan at its C terminus did not localize with endogenous LAMP-1 at all (Fig. 6) despite the fact that greater than 50% of the chimera molecules were intracellular. In cells expressing the C-terminal tryptophan chimera, prominent nuclear rings of immunolabeling with Mab-CV24 could be seen by conventional fluorescence microscopy of cells with and without cycloheximide treatment, suggesting that the protein was being retained in the endoplasmic reticulum (data not shown).

The remaining chimeras (C-terminal A, G, stop, and K) were detected only at the cell surface by immunofluorescence

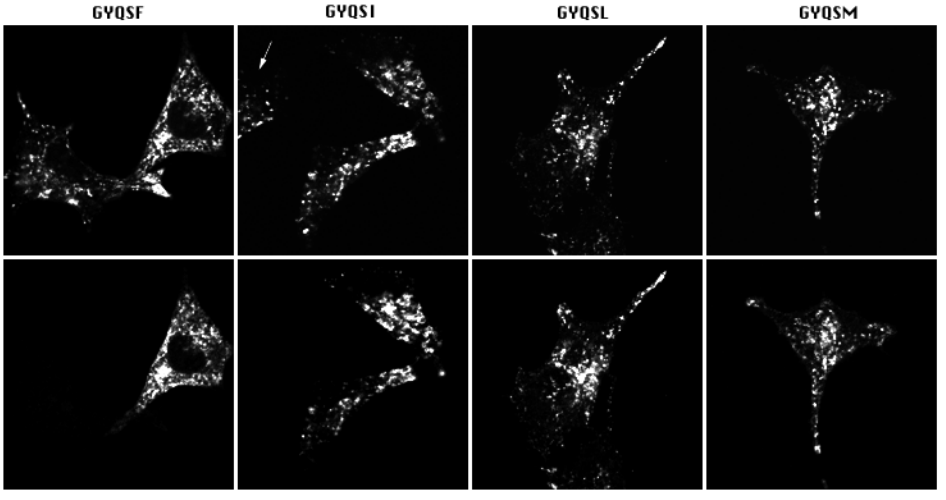

Fig. 4. Colocalization by confocal microscopy of LAMP chimeras having F,I,L or M at the C terminus with mouse LAMP-1. Cells were induced with butyrate, then treated with cycloheximide for 4 hours. After fixation and permeabilization, the cells were labeled with Cy3-Mab-1D4B (top row) and FITC-Mab-CV24 (bottom row). The arrows indicate non-transfected cells.

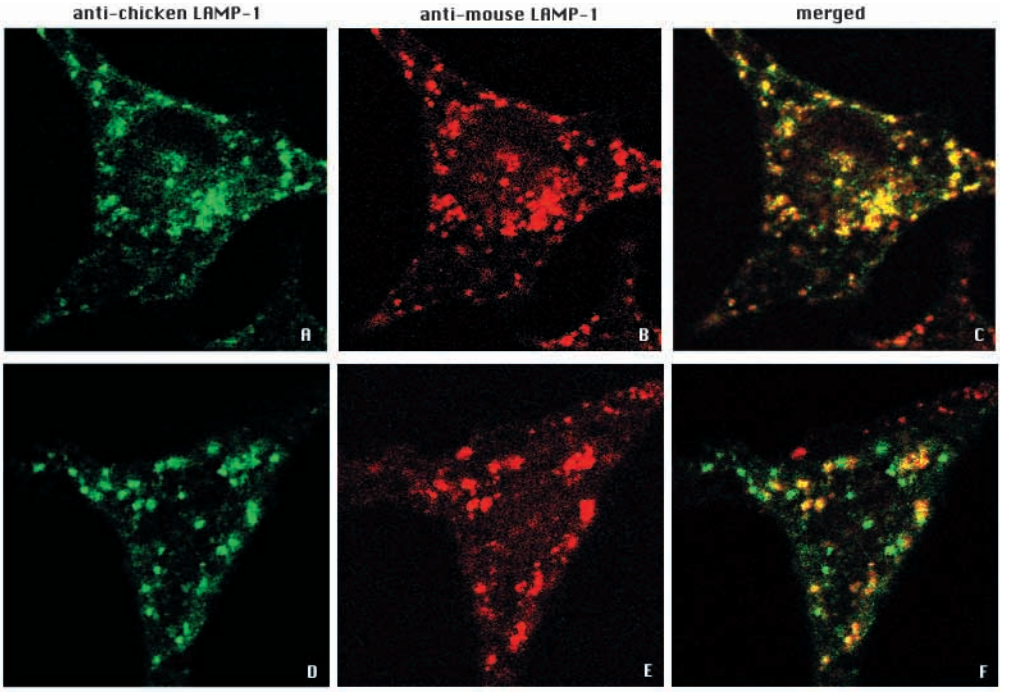

Fig. 5. Confocal microscopy of the intracellular expression of chimeras with Y (top row) or C (bottom row) at the C terminus. Cells were induced with butyrate, then treated with cycloheximide for 4 hours. After fixation, surface sites of the chimeras were blocked with unlabeled Mab-CV24, then the cells were permeabilized and labeled with FITC-Mab-CV24 (A,D) and Cy3-Mab-1D4B (B,E). The last panel in each row shows the merged images.

microscopy. A confocal image of a cell expressing the chimera with alanine at the C terminus is shown in Fig. 6. The plasma membrane immunofluorescent labeling is apparent, and there is no overlap with the pattern of labeling of the endogenous mouse LAMP-1 in lysosomes.

The C-terminal residue influences the utilization of the indirect pathway to the lysosome

LAMPs can reach the lysosome via two routes: (1) by direct transport from the trans-Golgi network to the late endosome and lysosome or (2) by the indirect pathway, appearing first at the plasma membrane and then being internalized and targeted to the lysosome from the endosomal compartment. In order to determine if the LAMP C-terminal residue influenced the ability of the LAMP chimeras to reach the lysosome from the plasma membrane, the ability of Mab-CV24 applied to the surface of cells to accumulate in lysosomes was determined by immunofluorescence microscopy. Uptake of this antibody has been shown to be mediated by binding to LAMP-1 in the plasma membrane (Lippincott-Schwartz and Fambrough, 1986, 1987). Mab-CV24 was added to the medium of the transfected cells for 30 minutes to 3 hours and the location of

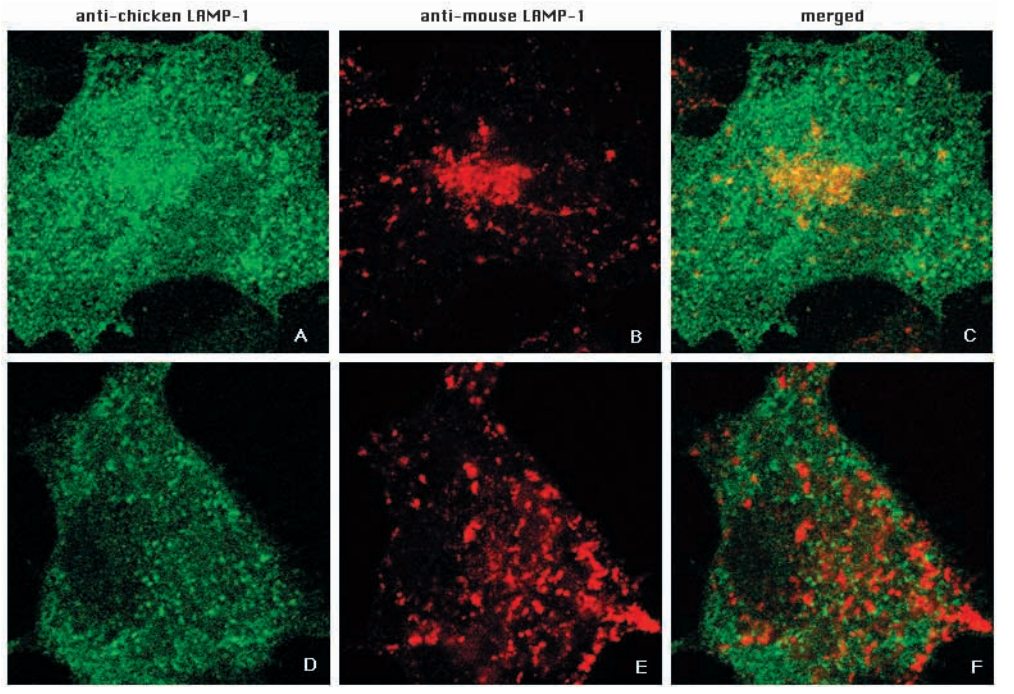

Fig. 6. The LAMP chimeras with C-terminal alanine (top row) and tryptophan (bottom row) do not colocalize with mouse LAMP-1 by confocal microscopy. Cells were induced with butyrate, then treated with cycloheximide for 4 hours. (A,B,C) A cell expressing the C-terminal alanine chimera which were fixed, permeabilized and labeled with FITC-Mab-CV24 (A) and Cy3-Mab-1D4B (B). (D,E,F) A cell expressing the chimera with C-terminal tryptophan, which, after fixation, were incubated with unlabeled Mab-CV24 to block surface sites of the chimera, then the cells were permeabilized and labeled with FITC-Mab-CV24 (D) and Cy3-Mab-1D4B (E). The last panel in each row shows the merged images.

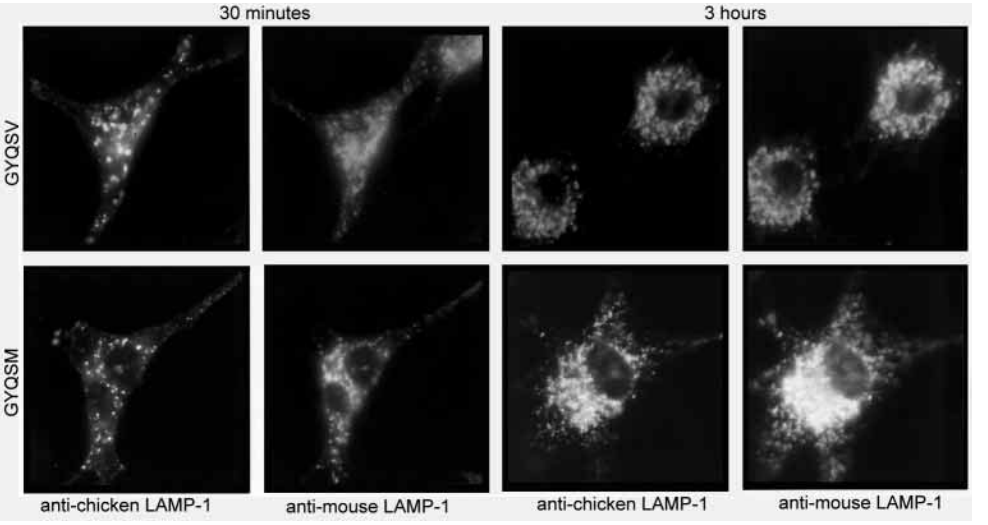

Fig. 7. Internalization of chimeras with C-terminal valine and methionine analyzed by immunofluorescence microscopy shows limited delivery to lysosomes at 30 minutes. Mab-CV24 was added to live, induced cells for 30 minutes or 3 hours. Cells were fixed, permeabilized and labeled with FITC-goat anti-mouse. Lysosomes were labeled with Cy3-Mab-1D4B. Scale is the same as Fig. 8.

Mab-CV24 was determined in fixed cells by indirect immunofluorescence microscopy and the location of lysosomes was identified by Mab-1D4b against endogenous LAMP-1. The chimeras again fall into two categories: one in which the Mab-CV24 accumulated in lysosomes over time and a second in which the Mab-CV24 remained at the cell surface.

LAMP chimeras ending in C-terminal residues found in naturally occurring LAMPs (V,L,I,F,M) all mediated accumulation of Mab-CV24 in lysosomes during the three hour time course (Figs 7 and 8). This was true both for chimeras in which the previously mentioned C-terminal residues were expressed on the LAMP-2b cytoplasmic tail and LAMP chimeras with the native avian LAMP-1, LAMP-2a and LAMP-2c cytoplasmic tails. After 30 minutes the uptake mediated by LAMPs with C-terminal methionine or valine resulted in accumulation of Mab-CV24 in a compartment near the cell periphery with very little overlap with lysosomes (Fig. 7), suggesting that these LAMPs were slower to transit out of the endosomal compartment once internalized. After 30 minutes of uptake, LAMPs with C-terminal phenylalanine, isoleucine, and leucine were present in both peripheral structures and in structures which colocalized with endogenous LAMP-1 (Fig. 8), indicative of a transit rate out of endosomes faster than C-terminal methionine and valine LAMPs. LAMP chimeras with C-terminal alanine, lysine, cysteine and tyrosine did not mediate internalization of Mab-CV24 detectably over the three hour time course (data not shown). These negative results demonstrate that non-specific uptake of antibody, for example by fluid phase endocytosis was negligible.

LAMP chimeras are internalized with different efficiencies

Qualitatively, LAMP chimeras with each of the naturally occurring cytoplasmic tail sequences were competent to mediate trafficking of LAMP antibodies to lysosomes. To determine if there are quantitative differences in the ability of the LAMPs with different C-terminal residues to utilize the indirect pathway, net internalization of iodinated Mab-CV24

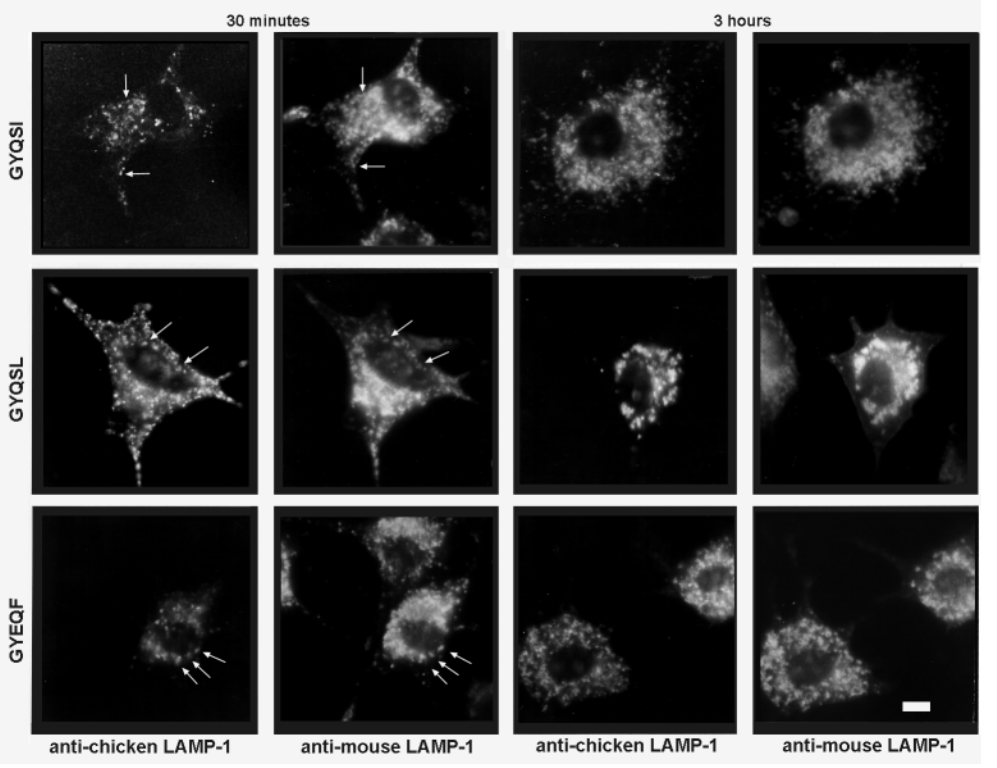

Fig. 8. Internalization of chimeras with C-terminal leucine, isoleucine and phenylalanine analyzed by immunofluorescence microscopy. Cells were processed as described in Fig. 7. Arrows indicated examples of the chimeras colocalizing with lysosomes at 30 minutes. Bar, 10 μm.

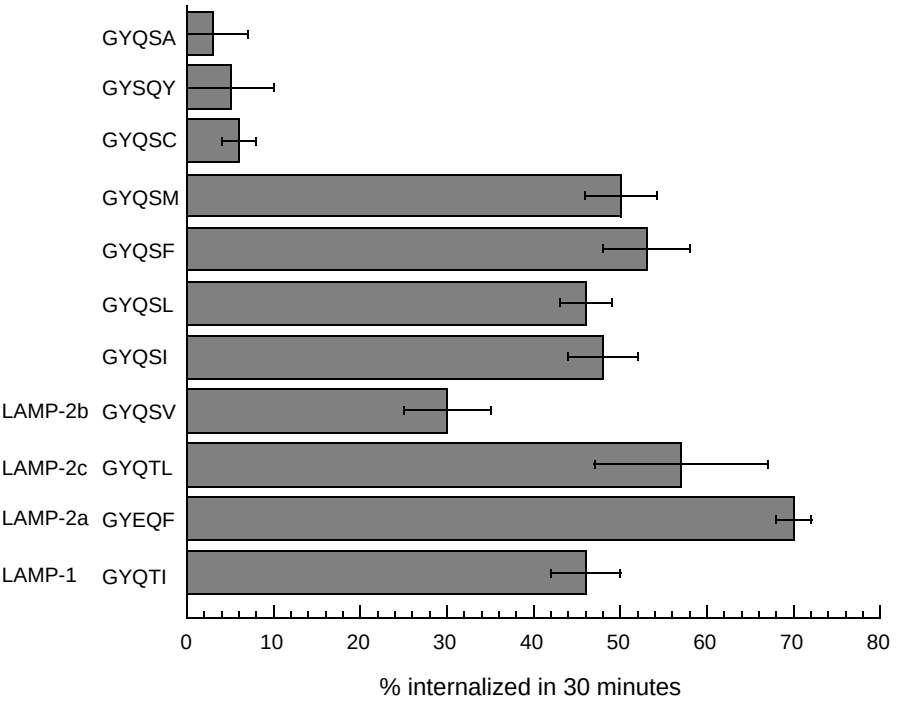

Fig. 9. Internalization of the LAMP chimeras in 30 minutes. The targeting signal of each chimera is indicated. Surface binding sites for ^{125}I-Mab-CV24 were labeled on live cells and the cells were incubated for 30 minutes at 37°C. Internalization was calculated from the amount of specifically bound ^{125}I-Mab-CV24 that could not be removed by acid stripping following the 30 minutes incubation period at 37°C, as described in Materials and Methods. The average of at least two experiments, each done in duplicate, and the standard deviation are shown.

was measured (Fig. 9). We hypothesized that the net amount internalized after 30 minutes at 37°C would be inversely related to the level of cell surface expression of the chimeras. This time point represents the steady-state between molecules internalized and those recycled to the plasma membrane (Lippincott-Schwartz and Fambrough, 1987; Gough and Fambrough, 1997). These studies were performed with all of the chimeric LAMPs that colocalized to any extent in lysosomes (C-terminal F, I, L,M, C, and Y and the chimeras containing the full length LAMP-2a, b and c tails). Wild-type avian LAMP-1 and the LAMP chimera with C-terminal alanine were included as positive and negative controls for internalization, respectively. Consistent with the immunofluorescence antibody uptake experiments, the chimeras with C-terminal cysteine, tyrosine, or alanine were negligibly internalized in 30 minutes (Fig. 9). The LAMP chimera with C-terminal valine was internalized to a moderate extent, while LAMP-1 and the other chimeras were internalized well in 30 minutes.

Efficient targeting of LAMPs to the lysosome correlates with the size of the C-terminal residue

To determine which factor (hydrophobicity or side chain size of the C-terminal residue) correlates most strongly with efficiency of lysosomal targeting, the targeting efficiency (expressed as percent of intracellular LAMP, a factor that generally correlated with lysosomal location) was plotted against two hydropathy scales and against the accessible surface area of the C-terminal side chain. The two scales for hydropathy are the Optimal Matching Hydropathy Scale (Sweet and Eisenberg, 1983) and the Kyte and Doolittle Hydropathy Index (Kyte and Doolittle, 1982). For the set of C-terminal residues in this study, there is essentially no correlation between efficiency of lysosomal targeting and hydropathy, using either scale (Fig. 10, upper panel). However, when accessible surface area of C-terminal residue is plotted against the percent of the LAMP chimera that is intracellular, the result is a bell-shaped curve (Fig. 10, lower panel). In the lower panel, the residues have symbols indicating whether or not they target LAMPs to lysosomes well, poorly or not at all. Based on the size correlation, an optimal LAMP targeting signal contains a C-terminal hydrophobic residue with side-chain surface area between 155 and 210 Å^2. From what is currently known about the targeting of LAMPs and other membrane proteins that contain a cytosolic Y-X-X-Φ signal, we expect that these requirements for LAMP trafficking reflect aspects of Y-X-X-Φ binding sites on adaptor complexes.

The C-terminal residue influences the ability of LAMP cytosolic tails to interact with adaptor subunits

During the trafficking of LAMP molecules through the cell, there are several places where the lysosomal targeting signal may be recognized: the trans-Golgi network, the endosomal compartments, and the plasma membrane. At each of these locations the lysosomal targeting signal may interact with an adaptor complex that could mediate recruitment of the chimera into coated membranes that subsequently bud from the compartment and result in vesicular transport of LAMP molecules to another compartment. Synthetic peptides resembling LAMP cytosolic tails have been demonstrated to interact with the plasma membrane adaptor complex AP-2 via the μ2 subunit (Ohno et al., 1995, 1996). Expression at the plasma membrane might be favored in those LAMP forms that are poorly internalized from the plasma membrane via AP-2 and clathrin mediated budding. The high level of cell surface expression of proteins with the LAMP-2b cytosolic tail was previously shown to be partly attributable to decreased internalization (Gough and Fambrough, 1997). Expression at the plasma membrane could also result from decreased

Table 2. Interactions between adaptor subunits and LAMP-1/LAMP-2b chimera targeting signals detected by growth on −His plates using the yeast two hybrid system

C-terminal sequence	β2	μ1	μ2	μ3A
GYQSV	-	-	-	±
GAQSV	-	-	-	-
GYQSA	-	-	-	-
GYQSI	-	-	+	++
GYQSL	-	-	++	++
GYQSM	-	-	-	+
GYQSF	-	-	++	++
GYQSC	-	-	-	-
GYQSY	-	-	-	+

The strength of the interaction (see Fig. 11) is indicated by the number of (+) symbols. No interaction is indicated by the (-) symbols.

Table 3. The strength of the interaction between LAMP-1/LAMP-2b targeting signals and adaptor subunits expressed as the IC_{50} (mM) for growth inhibition by 3AT

C-terminal sequence	β2	μ1	μ2	μ3A
GYQSV	-	-	-	-
GAQSV	-	-	-	-
GYQSA	-	-	-	-
GYQSI	-	-	0.04	10
GYQSL	-	-	21	10
GYQSM	-	-	-	0.1
GYQSF	-	-	21	10
GYQSC	-	-	-	-
GYQSY	-	-	-	0.03

The growth of yeast expressing the indicated fusion proteins in liquid media in the presence of the histidine synthesis inhibitor 3AT from 0.01 mM to 100 mM was determined. Only those which were positive for growth on His^- plates were tested (Table 2); (-) indicates not determined.

recognition of the targeting signal at the trans-Golgi network by the AP-1 μ1 subunit or by decreased recognition at the endosomal compartments, possibly via the AP-3 μ3A subunit, resulting in recycling of the protein to the plasma membrane. To determine if the LAMP-2b cytosolic tail mutants interacted with the μ subunits, the LAMP-2b mutants were tested for interaction with three μ subunits μ1, μ2 and μ3A in the yeast two hybrid system.

The LAMP-2b cytosolic tail and mutants thereof were fused to the GAL4 DNA binding domain with a sequence from TGN38 as a linker to enhance the interaction between the targeting signal and the adaptor μ subunits, which were fused to the GAL4 activation domain (Ohno et al., 1995, 1996). Positive interactions were measured as the ability to grow on histidine-deficient media as well as sensitivity to the histidine synthesis inhibitor 3AT (Fig. 11; Tables 2 and 3). Lack of interaction with the β2 subunit of the AP-2 complex and the dependence of the interaction on the critical tyrosine were used as controls for the specificity of the interactions (Fig. 11).

In Fig. 11, yeast expressing each of the test constructs were plated on histidine containing medium and histidine deficient medium to detect interactions between the LAMP-2b tail and mutants and the adaptor subunits. None of the sequences tested detectably interacted with β2 or μ1. Several of the contructs (C-terminal I, L and F) were capable of binding the AP-2 μ2 subunit, sequences ending in L and F having the strongest interaction (Table 3). An even larger subset of the constructs were recognized by the AP-3 μ3A subunit, sequences ending in I, L, and F showing the strongest interaction and V, M, and Y showing weaker interaction (Table 3). The μ3A interaction data correlate nicely with the immunofluorescent antibody uptake

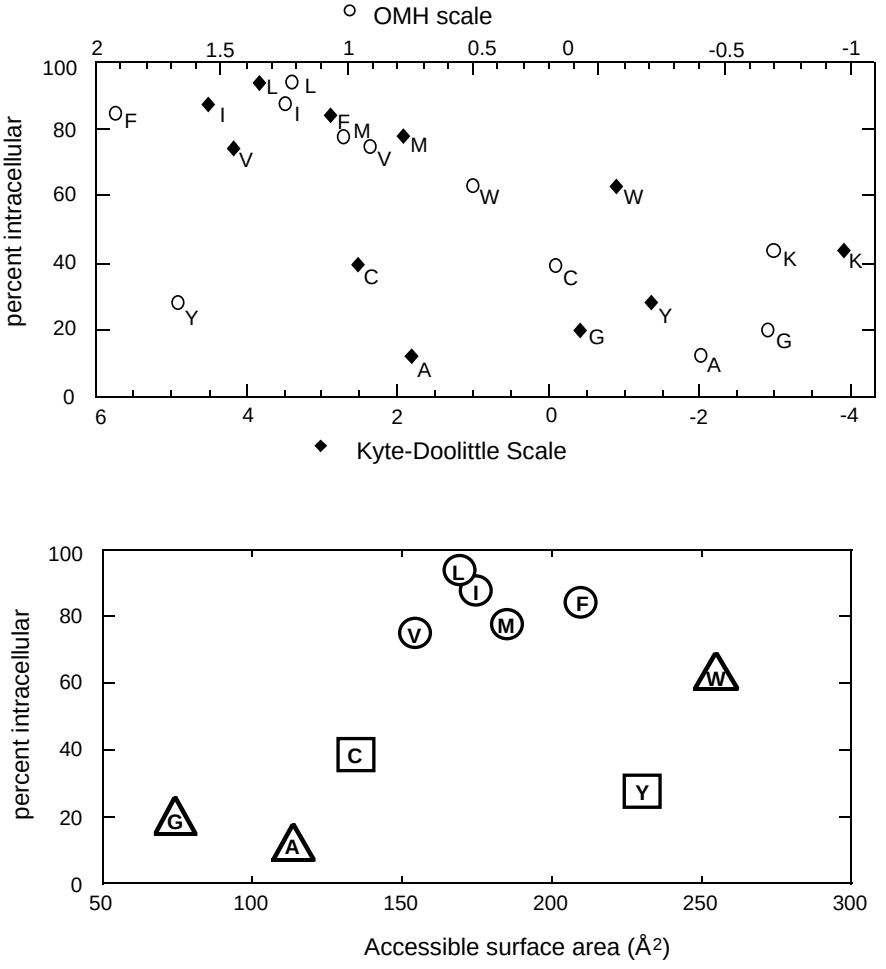

Fig. 10. Efficiency of lysosomal targeting compared to hydrophobicity based on OMH hydrophobicity scale (○) and Kyte-Doolittle hydropathy scale (◆) (A) and accessible surface area (B) of the C-terminal residue. Lysosomal targeting is indicated as the percent intracellular LAMP chimera, with the exception of the chimeras with C-terminal W, A, K or G. The C-terminal W, A, K and G chimeras did not accumulate at all in lysosomes. Data points for residues W, A and G are indicated by triangles in B. The LAMP chimeras with C-terminal C and Y (which remained predominantly at the cell surface but could accumulate to some extent in lysosomes) are indicated by squares in B.

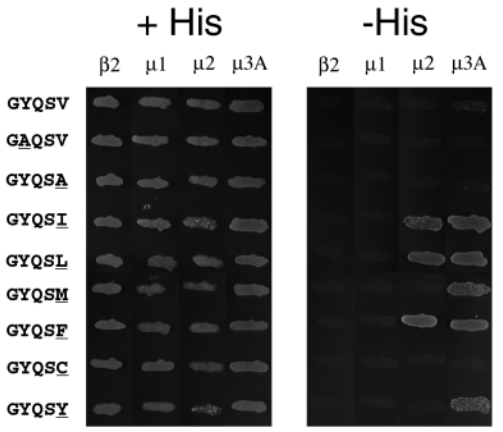

Fig. 11. Interaction of TGN38/LAMP-2 fusions with adaptor subunits tested by growth on histidine deficient plates. +His indicates plates containing histidine, –His indicates plates deficient in histidine. The targeting sequence (with changes from wild-type LAMP-2b underlined) tested is indicated to the left of each row and the adaptor subunit is indicated across the top.

experiments described above, in which LAMPs ending in V and M resided in endosomes longer while LAMPs ending in I, L, and F were more rapidly transported from the plasma membrane to lysosomes. Likewise, the interaction of the signal ending in tyrosine with μ3A suggests that LAMPs with this targeting motif may be able to reach lysosomes from the endosomal compartment at some rate. Thus, interactions with AP-2 μ2 and AP-3 μ3A are likely to explain the specific requirements of C-terminal residues for lysosomal targeting in vivo.

DISCUSSION

The present study represents the first systematic study of the requirements at the C-terminal position for lysosomal targeting signals of the '-G-Y-X-X-Φ' type. From the results of this study, several conclusions can be drawn. First, both hydrophobicity and side-chain size of the C-terminal residue are important in the -G-Y-X-X-Φ targeting signal. Second, the C-terminal residue influences specifically the internalization of LAMP molecules from the plasma membrane and trafficking out of the endosomal compartment, processes involved in setting the steady state distribution of LAMP molecules in the cell. Third, apparent affinities of lysosomal targeting sequences for adaptor μ chains, as inferred from yeast two-hybrid experiments, help to explain the subcellular distributions of the various LAMP forms. Finally, when lysosomal targeting is disrupted by alteration of the C-terminal residue, the mutant protein is not necessarily targeted exclusively to the plasma membrane, suggesting that some sorting machinery recognizes these altered signals and influences cellular distribution.

The consensus lysosomal targeting signal of the LAMP proteins shares features with other tyrosine based signals conforming to the consensus motif -Y-X-X-Φ-, which mediate rapid internalization, trans-Golgi network localization and basolateral targeting in polarized cells. The recognition of Y-X-X-Φ as a lysosomal targeting signal appears to involve (a) the presence of a tyrosyl (Williams and Fukuda, 1990) preceded by a critical glycyl residue (Hunziker et al., 1991; Harter and Mellman, 1992; Höning and Hunziker, 1995), (b) the presence of this -G-Y-X-X-Φ signal at the C terminus (Guarnieri et al., 1993), and (c) the occurrence of this C-terminal signal at a narrowly restricted distance from the lipid bilayer (Rohrer et al., 1996). Since the position of the signal at the C terminus is important, it was reasonable to expect that the C-terminal residue itself might play a role in lysosomal targeting. Indeed, in previous studies Guarnieri et al. (1993) reported that deletion of the C-terminal isoleucine from LAMP-1 or substitution of T for I disrupted lysosomal targeting, while substitution of L or F for I did not. Höning and Hunziker (1995) showed that an I to A substitution at the C terminus of LAMP-1 also disrupted targeting. Mutant LAMP-1 accumulated at the plasma membrane in these cases of disrupted targeting. In addition, we reported that the cell can discriminate among residues that occur naturally in the C-terminal position of lysosomal targeting signals of LAMPs (C-terminal V, I, L and F), leading to different distributions of the LAMP proteins between lysosomes and the cell surface (Gough et al., 1995; Hatem et al., 1995; Gough and Fambrough, 1997).

Both hydrophobicity and side chain size appear to play roles in C-terminal residue recognition

To better understand the nature of lysosomal targeting signals, we created a set of LAMP-2b targeting signal variants which ended in each of the hydrophobic residues as defined by either the OMH (optimal matching hydrophobicity) scale (Sweet and Eisenberg, 1983) or Kyte-Doolittle hydropathy index (Kyte and Doolittle, 1982). Obviously, the various hydrophobic residues are not equally effective in the lysosomal targeting signal (Fig. 10). C-terminal residue side chain size, as well as hydrophobicity, appears to contribute to recognition of the tyrosine-based signal for lysosomal targeting. When accessible surface area of C-terminal residue is plotted against the efficiency of lysosomal targeting conferred, the result is a bell-shaped curve. Considering these data and the fact that the chimera with C-terminal tryptophan does not target to lysosomes, we conclude that the optimal lysosomal targeting signal C-terminal residues have surface areas between 155 and 210 $Å^2$ (Fig. 10). Lysine is a bulky residue with a strongly hydrophobic surface out to the epsilon amino group, which bears a positive charge. The fact that terminal lysine prevents sorting to lysosomes suggests that the recognition site for the targeting signal involves a hydrophobic pocket to accommodate the C-terminal residue side-chain, rather than a superficial non-polar binding site.

It is important to note that operational definitions of hydrophobicity vary enormously and there is widely divergent opinion as to the hydrophobicity of certain amino acids employed in the present study. In our opinion, the OMH scale of hydrophobicities (Sweet and Eisenberg, 1983) seems most relevant for considerations of protein-protein interactions. The OMH scale reflects the frequency with which different aminoacyl residues occur as substitutions in the hydrophobic regions of proteins with evolutionarily conserved tertiary structure. These hydrophobic regions typically lie within the core of globular proteins and at hydrophobic surfaces where strong protein-protein interactions tend to occur, and one

can argue that the OMH scale reflects size as well as hybrophobicity in that it ranks the fit of hydrophobic residues into such positions. On the other hand the Kyte-Doolittle scale of hydropathies is based upon 'an amalgam of experimental observations derived from the literature' that include free energy of transfer from more polar to less polar phases as well as the distribution between surface and interior of proteins.

The residues that occur naturally in -G-Y-X-X-Φ lysosomal targeting sequences are the only residues in the present study that mediate efficient lysosomal targeting. All of these five residues fall within the set of moderate to highly hydrophobic on both the OMH and Kyte-Doolittle scales. However, the remaining hydrophobic residues illustrate difficulties in interpretation of the concept of hydrophobicity as applied to aminoacyl side-chains. Tyrosine is the second most hybrophobic residue on the OMH scale but ranks as slightly hydrophilic on the Kyte-Doolittle scale. If we imagine the recognition site for the lysosomal targeting signals as possessing a hydrophobic pocket to accommodate the C-terminal residue, residues that are too large as well as residues that are too small would be expected to associate poorly and thus not be targeted efficiently to lysosomes. In this way of conceptualization, C-terminal tyrosine may be too large. Tryptophan is even larger. It is slightly hydrophobic on the OMH scale; while its assigned hydrophobicity on the Kyte-Doolittle scale is simply the average of several disparate values of free energy change in partitioning experiments. Cysteine and alanine, on the other hand, may be a little too small to fit a binding pocket involved in targeting signal recognition. These two residues rank just above and below methionine, respectively, on the Kyte-Doolittle hydropathy scale, but unlike methionine, they do not mediate efficient lysosomal targeting. On the OMH scale, moreover, C and A have minimal or no hydrophobicity in terms of replacing other hydrophobic residues in the evolution of proteins with conserved tertiary structure. The OMH values of C and A may themselves reflect small size as well as hydrophobic nature.

One class of molecules that are excellent candidates for interactions with the LAMP targeting signal is the adaptors (Marks et al., 1996; Robinson, 1997; Odorizzi et al., 1998) which interact with tyrosine-based signals and mediate the formation of clathrin-coated membranes at the plasma membrane (AP-2), the trans-Golgi network (AP-1), and perhaps early endosomes (AP-3; Odorizzi et al., 1998). Recently, the μ2 subunit of AP-2 was crystallized bound to tyrosine based endocytosis signals, F-Y-R-A-L-M from the EGF receptor and D-Y-Q-R-L-N from the TGN38 protein, confirming a hydrophobic pocket for the residue three residues past the tyrosine, which is the position of our C-terminal residue in LAMPs (Owen and Evans, 1998). This hydrophobic binding pocket is defined primarily by Leu 175, Leu 173, Val 401, Leu 404, Val 422 and the aromatic residues Tyr 403 and Trp 421. Notably, two basic residues (Arg 402 and Lys 420) are at the edge of the pocket, suggesting a role for electrostatic interactions. While the crystallographic structure reveals the presence of the hydrophobic pocket opposite to residue five in the targeting peptide, the high temperature factors of the atomic coordinates preclude a quantitative computation of the expected binding order of targeting peptides with mutations at that position. With the information available at this point, it seems that the observed effect is correlated with the accessible surface area of the residue at position five. A surface area on the order of 90 $Å^2$ apparently provides maximal van der Waal contacts.

The medium subunits of the AP-1, AP-2 complexes interact in vitro with synthetic tyrosine-based sorting signals and show a strong dependence on the presence of a bulky hydrophobic residue at the C-terminal position of the sorting signal; however, in most cases the sorting signal in the native protein is not at the extreme C terminus of the sorted protein (Ohno et al., 1995, 1996). Such signals do occur at the very C terminus of the lysosomal membrane proteins LAMP-1, LAMP-2, LIMP-1, and CD68.

Interactions with the adaptor subunits μ2 and μ3 may mediate the targeting of LAMPs by the indirect pathway

LAMPs are internalized from the cell surface into coated vesicles by adaptor- and clathrin-mediated endocytosis (Lippincott-Schwartz and Fambrough, 1987). Subsequently, they are transported to endosomes and can either recycle to the plasma membrane or be transported to lysosomes, a process which may involve the AP-3 complex. Consistent with the idea that adaptors may play a role in the targeting of LAMPs and that their binding to the LAMP tail may be sensitive to the C-terminal residue, we found that the cells internalized the C-terminal mutant LAMP chimeras with different efficiencies. In general, the chimeras which were expressed at the lowest levels on the cell surface and which colocalized with endogenous LAMPs (chimeras with C-terminal F, I, L, and M) were internalized well, suggesting a functional interaction with the AP-2 complex. Those chimeras which were predominantly at the cell surface (C-terminal C, Y, and A) were internalized negligibly, indicating that their interactions with the AP-2 complex were poor. The fact that a small fraction of the chimeras ending in cysteine and tyrosine did colocalize with endogenous LAMP-1 in lysosomes but were not internalized efficiently suggests the possibility that at some step before reaching the plasma membrane the targeting signals of these chimeras were recognized as lysosomal targeting signals, albeit weakly. These observations in cells were supported by measurements of interactions of the tail sequences with the μ2 subunit of AP-2 in the yeast two hybrid system, in which LAMPs with C-terminal I, L and F interacted most strongly. Surprisingly, the LAMP chimera ending in M was internalized efficiently, yet did not interact with the μ2 subunit of AP-2 in the yeast two hybrid system, indicating that either the AP-2 complex does not mediate internalization of this chimera or that the display of this targeting signal by the two hybrid system is not optimal for recognition by the μ2 subunit.

In order to reach the lysosome from the plasma membrane the LAMPs must be sorted at the endosomes. The LAMP chimeras which were found competent to internalize by the quantititative assay all ultimately accumulated in lysosomes. However, the LAMPs with C-terminal V and M showed a prolonged residence in the endosomal compartment, suggesting that their targeting signals are recognized less efficiently at the endosome. Since AP-3 has been proposed to be localized to endosomes (Dell'Angelica et al., 1998), it is likely that interactions with AP-3 may determine sorting from this compartments towards lysosomes. Again the yeast two hybrid data support this conclusion, since sequences ending in

V and M showing weaker interactions with μ3A than those ending in I,L or F.

The cysteine and tyrosine signals provide tools for testing signal specificity at other LAMP sorting locations, for example at the trans-Golgi network, where AP-1 mediates sorting to late endosomes and lysosomes (Höning et al., 1996) and at the endosome where AP-3 may mediate sorting to the lysosome. Indeed the sequence with tyrosine at the C terminus was able to interact with μ3A in the yeast two hybrid system suggesting that some of the LAMPs with this targeting signal may reach the endosome and be sorted to the lysosome from there. The transit to the endosome is most likely to be from an intracellular site as the tyrosine mutant is not internalized from the plasma membrane at any detectable rate.

Another exception to the correlation between lysosomal localization and efficient internalization is the chimera with the LAMP-2b tail (C-terminal valine) which accumulated in lysosomes but was internalized with only moderate efficiency. Consistent with this observation, valine or smaller hydrophobic amino acids in the C-terminal position of tyrosine-based signals leads to a relatively weak or no interaction of the signal with the medium subunits of AP-1 and AP-2 in yeast two hybrid assays (Boll et al., 1996; Ohno et al., 1996). Data presented here and by Gough and Fambrough (1997) show that LAMP targeting signals which end in valine are expressed at the highest levels at the cell surface of any naturally occurring LAMP targeting signal, are internalized the least well (Gough and Fambrough, 1997), and reside in endosomes for prolonged periods when utilizing the indirect pathway. In the yeast two hybrid experiments the LAMP-2b signal ending with valine displays no detectable interaction with the AP-2 medium subunit and displays weak interaction with the AP-3 medium subunit complex, consistent with our conclusion that decreased internalization from the plasma membrane and increased recycling from the endosomal compartment contribute to higher cell surface expression of certain LAMP forms.

The correlation between in vivo targeting data and protein interaction analyses are generally consistent with the idea that both AP-2 and AP-3 participate in targeting LAMPs to lysosomes, AP-2 by mediating rapid internalization from the plasma membrane and AP-3 perhaps by mediating sorting from early to late endosomal/pre-lysosomal compartments. A role for AP-3 in lysosomal targeting has also been suggested by studies of trafficking in AP-3 deficient cells, which display increased trafficking of LAMPs through the plasma membrane (Le Borgne et al., 1998; Dell'Angelica et al., 1999). Additional studies are required to explore the extent to which LAMP-2b utilizes AP-1 for the direct route from trans-Golgi network to lysosomes. In the yeast two hybrid system, interactions of the LAMP targeting motifs with the μ1 subunit of AP-1 are weak even for strong targeting sequences such as the LAMP-1 cytoplasmic tail (Ohno et al., 1995). However, LAMP-1 has been demonstrated to interact with the intact AP-1 complex (Höning et al., 1996). The presence of a substantial amount of LAMPs in lysosomes of AP-3-deficient cells (Dell'Angelica et al., 1999) and the fact that LAMP-2 does not seem to interact well with the μ1 subunit of AP-1, raise the possibility that other molecules, yet to be identified, recognition may also play a role in lysosomal targeting.

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